Use of Sandwich-Cultured Human Hepatocytes to Predict Biliary Clearance of Angiotensin II Receptor Blockers and HMG-CoA Reductase Inhibitors

ABSTRACT:

Previous reports have indicated that in vitro biliary clearance (\(Cl_{biliary}\)) determined in sandwich-cultured hepatocytes correlates well with in vivo \(Cl_{biliary}\) for limited sets of compounds. The purpose of this study was 1) to determine the in vitro \(Cl_{biliary}\) in sandwich-cultured human hepatocytes of angiotensin II receptor blockers and HMG-CoA reductase inhibitors that undergo limited metabolism and 2) to compare the predicted \(Cl_{biliary}\) values with estimated in vivo hepatic clearance data in humans. The average biliary excretion index and in vitro intrinsic \(Cl_{biliary}\) values of olmesartan, valsartan, pravastatin, rosuvastatin, and pitavastatin in sandwich-cultured human hepatocytes were 35, 23, 31, 25, and 16%, respectively, and 0.943, 1.20, 0.484, 3.39, and 5.48 ml/min/kg, respectively. \(Cl_{biliary}\) values predicted from sandwich-cultured human hepatocytes correlated with estimated in vivo hepatic clearance values based on published data (no in vivo data in humans was available for pitavastatin), and the rank order was also consistent. In conclusion, in vitro \(Cl_{biliary}\) determined in sandwich-cultured human hepatocytes can be used to predict in vivo \(Cl_{biliary}\) of compounds in humans.

Many drugs, including cardiovascular, anticancer, and anti-infective agents (Bi et al., 2006; Shitara et al., 2006), undergo extensive biliary excretion in humans. Biliary excretion often plays an important role in the pharmacologic and pharmacokinetic behavior of these compounds. For example, enterohepatic recycling may prolong the pharmacologic effect of a compound by maintaining therapeutic concentrations for an extended period of time. In patients with renal impairment, biliary excretion may provide an alternative route of elimination, potentially avoiding elevated blood concentrations of drugs that might otherwise be excreted in the urine (Ishizuka et al., 1997; Nakagomi-Hagihara et al., 2006). Accordingly, knowledge regarding the extent of biliary excretion of compounds in humans during the early stages of drug development would be important in the drug discovery process. Elucidation of the biliary excretion properties of a drug candidate is also critical, considering the potential for drug-drug interactions and disease state alterations in hepatobiliary drug disposition.

In a recent study, we demonstrated that the in vitro \(Cl_{biliary}\) of angiotensin II receptor blockers (ARBs; olmesartan and valsartan) and HMG-CoA reductase inhibitors (statins; pravastatin, rosuvastatin, and pitavastatin) determined in sandwich-cultured rat hepatocytes can be used to estimate in vivo \(Cl_{biliary}\) and to ascertain the involvement of transport proteins in the basolateral uptake and canalicular excretion of these drugs (Abe et al., 2008). All of these compounds undergo limited metabolism and are organic anions with a carboxylic acid moiety, and they are excreted extensively into bile by canalicular transport proteins (Hirano et al., 2005; Nakagomi-Hagihara et al., 2006; Shitara and Sugiyama, 2006; Yamashiro et al., 2006). These compounds exhibited a wide range of biliary clearance values (low, olmesartan and valsartan; medium, pitavastatin; and high, rosuvastatin and pravastatin). The biliary clearance of the selected compounds should be governed by the unbound fraction and the hepatic uptake ability because these compounds were subject to minimal metabolism and exhibited uptake-limited hepatic clearance. Similar investigations in sandwich-cultured human hepatocytes would be useful in the preliminary evaluation of the hepatobiliary disposition of candidate compounds; data generated could provide the necessary information to 1) predict the rank order of in vivo \(Cl_{biliary}\) in humans, 2) assess the potential for drug-drug interactions in hepatobiliary transport, and 3) understand species differences in hepatobiliary disposition. Limited data are available for in vitro-in vivo correlations of the \(Cl_{biliary}\) of compounds due to the complexity of the procedures required to measure biliary excretion in humans (Ghibaillini et al., 2007).

The purpose of this study was 1) to determine in sandwich-cultured human hepatocytes the in vitro \(Cl_{biliary}\) of ARBs and statins that undergo limited metabolism and 2) to compare the predicted in vivo \(Cl_{biliary}\) values based on the in vitro data with estimated published in

ABBREVIATIONS: \(Cl_{biliary}\), biliary clearance; ARB, angiotensin II receptor blocker; CDFDA, 5 (and 6)-carboxy-2’,7’-dichlorofluorescein diacetate; CDF, 5 (and 6)-carboxy-2’,7’-dichlorofluorescein; HBSS, Hank’s balanced salt solution; BEI, biliary excretion index; AUC, area under the medium concentration-time curve; MRP, multidrug resistance-associated protein; OATP, organic anion-transporting polypeptide.
vivo hepatic clearance data. In this study, hepatic clearance values for these drugs were estimated based on the difference between total clearance and renal clearance in humans.

Materials and Methods

Chemicals. Olmesartan was kindly provided by Daiichi-Sankyo Co., Ltd. (Tokyo, Japan). Valsartan, pravastatin, rosuvastatin, and pitavastatin were obtained from Toronto Research Chemicals Inc. (Ontario, Canada). $[^3]$H]-Taurocholate (5 Ci/mmol; purity >97%) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Dubecco’s modified Eagle’s medium, minimum essential medium nonessential amino acids, and 5 (and 6)-carboxy-2,7’-dichlorofluorescein diacetate (CDFDA) were purchased from Invitrogen (Carlsbad, CA). Insulin/transferrin/selenium culture supplement and Matrigel were purchased from BD Biosciences Discovery Labware (Bedford, MA). Penicillin-streptomycin solution, taurocholic acid, dexamethasone, and Matrigel were purchased from BD Biosciences Discovery Labware (Bedford, MA). Penicillin-streptomycin solution, taurocholic acid, dexamethasone, and Matrigel were purchased from BD Biosciences Discovery Labware (Bedford, MA). Insulin/transferrin/selenium culture supplement and Matrigel were purchased from BD Biosciences Discovery Labware (Bedford, MA).

Hepatocyte Culture. Freshly isolated human hepatocytes (seeding density: 1.5 million cells per well) overlaid with Matrigel in 6-well plates were provided by CellzDirect (Durham, NC). The demographics of the human liver donors are shown in Table 1. Hepatocytes were cultured in Dulbecco’s modified Eagle’s medium containing 1% insulin/transferrin/selenium, 0.1 μM dexamethasone, 2 mM L-glutamine, 1% minimum essential medium nonessential amino acids, 100 units of penicillin G sodium, and 100 μg per ml of streptomycin sulfate at 37°C in a humidified incubator with 95% O₂/5% CO₂ for 7 to 10 days; medium was changed daily.

Fluorescence Microscopy. Retention of 5 (and 6)-carboxy-2,7’-dichlorofluorescein (CDF) in bile canicular networks was examined by fluorescence microscopy. Hepatocytes were rinsed with 2 ml of standard buffer and then 1.5 ml of CDFDA (10 μM) in standard buffer was added. After a 10-min incubation at 37°C, the buffer was removed and 1.5 ml of standard buffer was added. The cells and bile canaliculi were imaged with a Zeiss Axiovert 100 TV inverted fluorescence microscope (Carl Zeiss Inc., Thornwood, NY).

Accumulation Studies and Analysis. Hepatocytes were rinsed twice and then preincubated for 10 min at 37°C with 2 ml of warmed Hank’s balanced salt solution (HBSS) containing Ca²⁺-free (standard; cells + bile) or Ca²⁺-free HBSS (cells), to maintain or disrupt the tight junctions sealing bile canicular networks, respectively. Subsequently, hepatocytes were incubated with test compound (5 μM for ARBs or statins; 1 μM for $[^3]$H]-Taurocholate) in standard HBSS for 10 min at 37°C. After incubation, the dosing solution was aspirated from the cells and uptake was stopped by washing the cells 3 times with ice-cold standard HBSS. For $[^3]$H]Taurocholate, cells were lysed with 1 ml of Triton X-100 (0.5%) in phosphate-buffered saline. For ARBs and statins, cells were lysed with 1 ml of methanol (70%; v/v) and sonicated for 20 s with a sonic dismembrator (model 100; Thermo Fisher Scientific, Waltham, MA) then stored at < −70°C until analysis. The samples were analyzed for drug concentrations by liquid scintillation counting or by liquid chromatography with tandem mass spectrometry, as described previously (Abe et al., 2008).

Substrate accumulation was corrected for nonspecific binding by using Matrigel-precoated dishes without cells. The total protein concentration in cell lysates was quantified by the BCA protein assay (Pierce Biotechnology, Inc., Rockford, IL) using bovine serum albumin as the reference standard, and accumulation was normalized to protein concentration. Due to incompatibility of the protein assay with methanol, the average protein concentration for samples lysed with Triton X-100 incubations in the same liver preparation was used to normalize accumulation.

Data Analysis. The accumulation (pmol/mg protein), biliary excretion index (BEI%), and in vitro intrinsic Clbiliary (ml/min/kg) were calculated in hepatocytes using B-CLEAR technology (Qualyst, Inc., Raleigh, NC) based on eqs. 1 and 2 (Liu et al., 1999a).

\[
\text{BEI} = \frac{\text{Accumulation}_{\text{cells} + \text{bile}} - \text{Accumulation}_{\text{cells} + \text{bile}}}{\text{Accumulation}_{\text{cells} + \text{bile}}} \times 100
\]

(1)

\[
\text{Intrinsic \ Cl}_{\text{biliary}} = \frac{\text{Accumulation}_{\text{cells} + \text{bile}} - \text{Accumulation}_{\text{cells} + \text{bile}}}{\text{AUC}_{\text{medium}}}
\]

(2)

where AUCmedium was determined as the product of the incubation time and the medium concentration. The concentration of drug in the medium was defined as the initial substrate concentration in the incubation medium, because the medium concentration at the beginning of incubation did not differ by more than 10% from the medium concentration at the beginning of incubation. In the absence of exogenous protein in the incubation medium, unbound intrinsic Clbiliary (intrinsic Cl′ biliary) was assumed to be equivalent to in vitro intrinsic Clbiliary (eq. 2). The in vitro intrinsic Clbiliary (ml/min/mg protein) was scaled to kilogram of body weight assuming the following: 1 mg protein/1.5 × 10⁸ hepatocytes/g human liver tissue (Wilson et al., 2003), and 25.7 g of liver tissue per kilogram of body weight (Davies and Morris, 1993). The predicted in vivo Clbiliary values were estimated according to the equations below based on the well-stirred model of hepatic disposition, assuming that red blood cell partitioning of test compounds was minimal.

\[
\text{Predicted Cl}_{\text{biliary}} = \frac{Q_p \times fu_p \times \text{intrinsic Cl}_{\text{biliary}}}{Q_p + fu_p \times \text{intrinsic Cl}_{\text{biliary}}}
\]

(3)

where Qp and fu_p represent the hepatic plasma flow rate and plasma unbound fraction, respectively. Hepatic plasma flow in humans (750 ml/min) (Sandker et al., 1994) was normalized by assuming a body weight of 70 kg.

The hepatic clearance of olmesartan in humans was reported to be 0.86 l/h based on total and renal clearance after intravenous administration (Nakagomi-Hagihara et al., 2006). The renal clearance of valsartan was estimated as the product of the total clearance (2.19 l/h) and the fraction excreted unchanged in urine after an intravenous dose (0.29) (Flesch et al., 1997). Likewise, the renal clearance of rosuvastatin was estimated from the total clearance (48.9 l/h) and the fraction excreted unchanged in urine (0.28) (regulatory documentation). Total clearance and renal clearance of pravastatin after intravenous administration in humans were reported to be 13.5 and 6.3 ml/min/kg, respectively (Singhi et al., 1990). Hepatic clearance values of these compounds in humans were estimated based on the difference between total clearance and renal clearance using the above information, assuming a body weight of 70 kg. No information regarding the total and renal clearance of pitavastatin after intravenous administration was available.

Results and Discussion

CDF retention in bile canaliculi of sandwich-cultured human hepatocytes was evaluated to assess canicular excretory function of the hepatocytes. CDFDA readily diffuses into hepatocytes, where it is hydrolyzed rapidly to CDF, a fluorescent compound that is extensively excreted into the bile canaliculi by multidrug resistance-associated protein (MRP)2 (Hoffmaster et al., 2004). CDF fluorescence accumulated in bile canicular networks of sandwich-cultured human hepatocytes after a 10-min incubation with CDFDA (Fig. 1). $[^3]$H]-Taurocholate accumulation in standard buffer (101–205 pmol/mg protein) and BEI (52.4–70.8%), equivalent to the percentage of retained substrate in the canicular networks, were in good agreement with

<table>
<thead>
<tr>
<th>Liver Identification</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Race</th>
<th>Comedications</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>57</td>
<td>Female</td>
<td>African American</td>
<td>Metoclopramide, docusate, acetaminophen, hydrocodone</td>
</tr>
<tr>
<td>2</td>
<td>64</td>
<td>Female</td>
<td>White</td>
<td>Atorvastatin, alendronate, amiodipine, benazepril</td>
</tr>
<tr>
<td>3</td>
<td>51</td>
<td>Male</td>
<td>White</td>
<td>Glyburide, metformin, pravastatin, bevacizumab, pioglitazone</td>
</tr>
</tbody>
</table>

TABLE 1

Demographics of human liver donors
The rank order of the predicted Cl_{biliary} values was consistent with the estimated in vivo hepatic clearance values for these drugs, although both ARBs have low in vivo hepatic clearance values. Both the predicted and the estimated in vivo hepatic clearance values of rosuvastatin were the highest, followed by pravastatin, valsartan, and olmesartan (Fig. 3; Table 2). Based on this study, the in vivo Cl_{biliary} of pitavastatin was predicted to be much lower than pravastatin and rosuvastatin. Although the bioavailability of pitavastatin is unknown in humans because no intravenous formulation is available, the oral clearance of pitavastatin in humans was reported to be lower than pravastatin and rosuvastatin (Ieiri et al., 2007).

The predicted Cl_{biliary} values were 5- to 30-fold lower than the estimated in vivo hepatic clearance values for ARBs and statins (Table 2). These findings may be due to less extensive canalicular network formation in culture compared with liver tissue in vivo. Consistent underestimation also may be due to several factors including, but not limited to, decreased activity of transport proteins in culture or leakage from the biliary compartment in sandwich-cultured hepatocytes, as discussed previously (Liu et al., 1999b; Hoffmaster et al., 2005). A recent study with hepatocytes demonstrated a significant underprediction of in vivo clearance for a distinct set of drugs, which was attributed to hepatic uptake (Riley et al., 2005). It is probable that a scaling factor might be necessary for the prediction of in vivo biliary clearance. Although unlikely, it is possible that the underestimation of Cl_{biliary} is due to more extensive metabolism of these compounds than previously recognized. According to the information on the Food and Drug Administration website, the contribution of metabolic clearance to total clearance for olmesartan, valsartan, and rosuvastatin in humans is quite low; these drugs are recovered primarily as unchanged species, with at most ~20% of the dose recovered as metabolites. Pravastatin is highly hydrophilic [LogD at pH 7 = −0.47], undergoes minimal metabolism by cytochrome P450 (Shitara and Sugiyama, 2006), and the contribution of metabolism to pravastatin clearance should be quite low.
Bi et al. (2006) reported that sandwich-cultured human hepatocytes prepared from cryopreserved human hepatocytes form intact bile canalicular networks and exhibit functional uptake and transporter-mediated excretion. Use of cryopreserved human hepatocytes would be more convenient than isolating and preparing sandwich-cultured human hepatocytes from fresh human liver. The reported BEI of taurocholate (41–63%) (Bi et al., 2006) was consistent with our results (53–71%), although the published in vitro intrinsic Cl_biliary of taurocholate (5.8–10 µl/min/mg protein) (Bi et al., 2006) was slightly lower than our results (12–25 µl/min/mg protein). However, the BEI (43–58%) and the in vitro intrinsic Cl_biliary (4.0–12 µl/min/mg protein) of rosuvastatin (Bi et al., 2006) was higher than our results (BEI, 17–33%; in vitro intrinsic Cl_biliary, 1.2–2.8 µl/min/mg protein). These differences may be attributed to differences in the type of medium, hepatocytes, or the day in culture when the experiments were performed (day 5 versus days 7–10). We previously confirmed that sandwich-cultured human hepatocytes cultured for 7 to 10 days maintain good morphology, form distinct bile canalicular networks, and express functional transport proteins on the proper membrane domain (Hoffmaster et al., 2004).

**FIG. 2.** Accumulation [cells + bile (solid bars) and cells (white bars)] and BEI of taurocholate (A), olmesartan (B), valsartan (C), pravastatin (D), rosuvastatin (E), and pitavastatin (F) in sandwich-cultured human hepatocytes. Dosing concentration: taurocholate (1 µM), ARBs and statins (5 µM); incubation time: 10 min. Data represent mean ± S.D. (triplicate).
In sandwich-cultured rat hepatocytes, the average in vitro intrinsic 
Cl\textsubscript{biliary} of olmesartan, valsartan, pravastatin, rosuvastatin, and pitavastatin 
was 0.215, 0.405, 0.553, 2.076, and 0.794 ml/min/mg protein, respectively, 
compared with values determined in sandwich-cultured primary rat and 
human hepatocytes. For example, transport properties of human OATPs 
and bile acid transporters in pitavastatin hepatic uptake: function, expression, 
and pharmacogenetics. Gastroenterology 130:1793–1806.


and bile acid transporters in rosuvastatin hepatocytes: function, expression, and pharmacogenetics.

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uptake by human hepatocytes of pitavastatin, a new inhibitor of HMG-CoA reductase.

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Address correspondence to: Dr. Kim L. R. Brouwer, University of North Carolina School of Pharmacy, Kerr Hall, CB#7360, Chapel Hill, NC 27599-7360. E-mail: kbrouwer@unc.edu